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Methodological review

Methods for isolating atrial cells from large mammals and humans

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ABSTRACT

The identification of disturbances in the cellular structure, electrophysiology and calcium handling of atrial cardiomyocytes is crucial to the understanding of common pathologies such as atrial fibrillation. Human right atrial specimens can be obtained during routine cardiac surgery and may be used for isolation of atrial myocytes. These samples provide the unique opportunity to directly investigate the effects of human disease on atrial myocytes. However, atrial myocytes vary greatly between patients, there is little if any access to truly healthy controls and the challenges associated with assessing the in vivo effects of drugs or devices in man are considerable. These issues highlight the need for animal models. Large mammalian models are particularly suitable for this purpose as their cardiac structure and electrophysiology are comparable with humans. Here, we review techniques for obtaining atrial cardiomyocytes. We start with background information on solution composition. Agents shown to increase viable cell yield will then be explored followed by a discussion of the use of tissue-dissociating enzymes. Protocols are detailed for the perfusion method of cell isolation in large mammals and the chunk digest methods of cell isolation in humans.

Contents

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1. Introduction

Disease of the cardiac atria is a large and growing global problem. Atrial fibrillation (AF) is the most prevalent arrhythmia in the developed world, affecting 33 million patients globally [1]. Current pharmacological options have imperfect efficacy and substantial adverse side-effects, including drug-induced proarrythmia and both cardiac and non-cardiac toxicity [2–4]. The limited effectiveness of current pharmacology is likely to result from an incomplete understanding of the pathophysiology of this complex heart rhythm disorder. Understanding the processes underlying AF requires investigation at several scales. Whilst studies at a whole-animal, whole-organ and tissue level have provided many insights into the origins of arrhythmia initiation and perpetuation [5], elucidating the pathological changes in ion currents, Ca^{2+} handling, and cellular microstructure requires isolated cardiomyocytes.

Much of our understanding of myocyte physiology has been obtained from rodent models [6–8], from which cell isolation has recently been reviewed [9]. Human electrophysiology, however, differs in several fundamental regards from these species. Examples of this include a five to ten-fold difference in resting heart rate [10], a markedly different complement of ion channels [11] and the presence of some transverse tubules in the atria of large mammals [12–14]. To this end, a potentially more fertile ground for understanding human disease is investigating cardiomyocytes obtained from humans or other large mammals.

This review aims to find the common thread that runs through the atrial isolation protocols that have been evolving for more than 30 years [15]. There is a paucity of papers directly comparing isolation techniques [16] and therefore the method sections from individual citations have been compared. A representative paper describing the protocol used by each research group can be found in the supplemental materials.

The theory underlying atrial myocyte isolation will be reviewed, and factors shown to influence cell yield and quality discussed. Some reagents, whilst improving cell quality for a specific experiment, may be detrimental to the requirements of a different investigation. Knowledge of these principles will therefore allow the isolation protocol to be tailored to the experiment in hand. This will be followed by examples of protocols that have proved successful for obtaining myocytes from humans and large mammals.

1.1. Search strategy

Examples of protocols were found by searching PubMed using the terms “atrial myocytes” OR “atrial cells” OR “heart cells” from 1st October 1980 to 1st April 2015, written in English and excluding reviews. Human studies were found by filtering for “human”, whilst reports on large mammalian species were found using the additional search terms of “dog” OR “canine”, “sheep” OR “ovine”, “goat”, “pig” OR “porcine” OR “swine” NOT “guinea pig”. References from key review articles on atrial electrophysiology were also identified.

2. Saline solutions

The basic composition of all solutions used for cell isolation is usually based on established physiological saline solutions. Historically, these were formulated by empirically adjusting ion concentrations until the solutions were considered acceptable in terms of the behaviour and survival of a specific tissue. Physiological salines which are commonly used in experimental cardiac research are shown in Table 1.

In his famous publications from the early 1880s Sydney Ringer described for the first time the importance of proper extracellular ion concentrations for the physiological function of the heart [17], establishing the importance of Na^+, K^+ and Ca^{2+} ions to maintain continuous beating of an isolated frog heart. To prevent acidification by metabolic products, he included bicarbonate buffers to maintain the cardiac contractions for longer periods. Later Locke [18] adapted Ringer's solution to the mammalian heart, by increasing salt concentrations. Locke also added 5.6 mmol/L glucose (0.1%) which substantially increased the survival time of the heart. Maurice Tyrode's widely used saline was originally developed to maintain contractions of isolated rabbit intestine [19]. He added magnesium and improved buffering by adding phosphate and increasing the concentration of bicarbonate.

Krebs and Henseleit performed experiments on liver sections [20]. They further adapted available physiological salines to resemble the composition of mammalian plasma by including sulphate and increasing the concentration of phosphate. They used higher concentrations of bicarbonate and gassed their solution with carbogen (5% CO_2, 95% O_2) to achieve a pH of 7.4.

Many of the physiological salines used today are based on the formulations described above. Usually more modern buffers, such as HEPES (*4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid*) or MOPS (3-(N-morpholino) propanesulfonic acid) are added to help maintain constant pH as their pK_a values are close to normal blood pH. Furthermore metabolic substrates such as pyruvic acid, β-hydroxybutyrate, glutamic acid, oxalic acid or succinate may be included in addition to glucose. Bovine serum albumin (BSA, 0.1–7%) is commonly used in experimental solutions to maintain colloid osmotic pressure, thereby preventing oedema [21], and to reduce nonspecific binding of proteins and toxins used for experimental research [22]. At higher concentrations, BSA is also used to inhibit enzyme activity [23].

These physiological salines have been optimised for different stages of the isolation process. The following section will describe the typical solutions used for (i) cardioplegia and tissue transport, (ii) Ca^{2+}-free solutions used for tissue dissociation, and (iii) storage solutions that are used once single myocytes have been obtained.

Table 1
Physiological salines used in cardiac research (in mmol/L) [118].

	Ringer [17]	Locke [18]	Tyrode [19]	Krebs and Henseleit [20]
NaCl	116	154	137	117
KCl	1.2	5.6	2.7	4.7
$CaCl_2$	1	2.1	1.8	2.5
$MgSO_4$				1.2
$MgCl_2$			1.1	
$NaHCO_3$	2.7	2.4	11.9	24.8
NaH_2PO_4			0.4	
KH_2PO_4				1.2
Glucose		5.6	5.6	11.1
pH				7.4
CO_2				5%

3. Cardioplegic solutions

Cardioplegic solutions are used during cardiac surgery to halt myocardial contraction, referred to as diastolic arrest. This decreases myocardial metabolic demands and slows the degenerative processes that follow ischaemia. The composition of commonly used transport solutions is comparable to cardioplegic solutions used for cardiopulmonary bypass surgery. The usage of cardioplegic solutions is almost ubiquitous in human work (Supplemental Table 4 and [24–33]), and these solutions are also used by some groups for isolation of atrial myocytes from large mammals [34–36].

Cardioplegic solutions are subdivided into extracellular vs. intracellular solutions based on their relative concentrations of Na^+, Ca^{2+} and K^+. Extracellular type solutions contain physiological Na^+ and Ca^{2+}, achieving diastolic arrest through hyperkalemia resulting in depolarisation of the cell membrane. St. Thomas solution no II, the most widely used cardioplegic solution in cardiac surgery, was successfully applied as transport medium for atrial specimens [37]. Its formulation, which is based on Ringer's solution, to which potassium chloride and magnesium chloride were added, originates from the St. Thomas Hospital in London [38].

Intracellular type cardioplegic solutions have a low Na^+ and/or Ca^{2+} content, similar to that which is found in the cell cytoplasm. Diastolic arrest is caused by offsetting of the transsarcolemmal Na^+ gradient, thereby reducing the driving-force of the fast Na^+ current responsible for the upstroke of the cardiac action potential. Reduced Ca^{2+} concentrations limit contraction and reduce ischaemia-induced Ca^{2+} entry. Bretschneider solution, a commonly used commercially available intracellular cardioplegic solution, contains histidine, tryptophan and ketoglutarate and was used by Hoppe and Beukelmann as transport medium for atrial tissue samples [39]. The low-Ca^{2+} solutions used during enzymatic digestions (see below) can also be seen as part of the family of intracellular cardioplegic solutions, and can therefore also be used as transport solution.

In addition to chemically-induced diastolic arrest, hypothermia reduces myocardial oxygen demands and slows cellular degeneration during transport. In addition, low temperatures may also reduce the detrimental effects of the Ca^{2+}paradox (see below). Whilst transport at 4 °C is used by some groups [34,36], we have not in our experience found a clear advantage to cooling or oxygenation with transport times between 30 and 45 min with respect to both number and quality of isolated atrial cardiomyocytes. In general transport to the lab should be as quick as possible. If longer transportation time cannot be avoided, transport at 4 °C in oxygenated solution may be advantageous.

4. Ca^{2+}-free solutions and the Ca^{2+} paradox

Once material has been transported to the laboratory, a separate solution is often used to dissociate the tissue. One common feature of all protocols used for isolation of single myocytes is a period of perfusion with a nominally Ca^{2+}-free solution (low Ca^{2+} solution). Even the earliest reports of successful isolation of myocytes from adult animals take advantage of Ca^{2+}-free buffers. It is assumed that this Ca^{2+}-free period is necessary to allow cell separation at the intercalated discs [40]. This may occur via disruption of the Ca^{2+}-dependent cadherins that mediate adherens and desmosomal junctions between myocytes [41]. Ca^{2+}-free perfusion also causes separation of the layers at the cell surface (external lamina from myocyte surface coat [42]), which may lead to the surface 'blebs' occasionally seen on isolated cells.

However, perfusion of either cardiac myocytes or the intact heart with Ca^{2+}-free solution has a major disadvantage first described by Zimmerman and Hulsmann [43]. They observed that after exposure of the heart to Ca^{2+}-free solution, reintroduction of Ca^{2+} into the perfusion fluid causes tissue disruption with marked contracture and release of intracellular enzymes. This phenomenon is known as the "calcium paradox" and results in irreversible hypercontraction of over 50% of isolated myocytes [44]. The Ca^{2+} paradox is assumed to be caused by an excessive increase of intracellular Ca^{2+} resulting in massive release of enzymes, exhaustion of tissue high-energy phosphates, and ultrastructural damage including contracture of myofilaments and mitochondrial swelling (For review see Piper 2010 [45]).

There are two main hypotheses regarding the mechanisms underlying the Ca^{2+} paradox. (1) The partial cellular uncoupling during Ca^{2+}-free perfusion means that myocyte contractile force is focused solely on the residual adherent areas during Ca^{2+} reintroduction [46]. This leads to mechanical damage through sarcolemmal perforation, massive Ca^{2+} influx, and sarcolemmal disruption. (2) The sarcolemma becomes abnormally permeable to electrolytes and/or water during Ca^{2+}-free perfusion, potentially due to Na^+ loading via Na^+/Ca^{2+} exchanger. Upon Ca^{2+} reintroduction, uncontrolled Ca^{2+} influx ensues, either through reverse Na^+/Ca^{2+} exchanger [47] or via transient receptor potential cation (TRPC) channels [44].

Protecting myocytes from the deleterious effects of the Ca^{2+} paradox has been suggested to increase the quality and quantity of isolated cells. The following section details interventions that have been suggested to increase Ca^{2+}-tolerant myocyte yield.

4.1. Duration of Ca^{2+}-free perfusion

Contraction is curtailed in the absence of Ca^{2+}, and upon reperfusion with solutions containing Ca^{2+}, a regeneration of contractile force is seen as a marker of myocyte health. In rat hearts, exposure to greater than five minutes of Ca^{2+}-free medium completely abolished force regeneration while shorter exposures did not [48]. This suggests that limiting exposure to Ca^{2+}-free medium may have a beneficial effect on the isolation of cardiac myocytes. Therefore Lalevee et al. [49] and Varro et al. [50] performed the enzymatic digestion in the presence of Ca^{2+} (10 μmol/L and 300 μmol/L, respectively) and introduced a washing step with nominally Ca^{2+}-free solution in between. Varro et al. clearly state that too brief exposure to the nominally Ca^{2+}-free solution probably failed to cause the intercellular junctions to disconnect, while too long an exposure yielded myocytes that were intolerant of Ca^{2+}.

4.2. Temperature and pH

The Ca^{2+} paradox is strongly dependent on temperature. Perfusion with Ca^{2+} free solution at 4 °C, followed by the restoration of Ca^{2+}, permitted recovery of cardiac function upon warming and protection of cellular organelles while a constant temperature of 37 °C throughout the procedure led to irreversible myocytolysis [51]. It is assumed that the reduced temperature prevents separation of the fuzz-like glycoproteins that cover the cell membrane (the glycocalyx) and limits cytosolic Ca^{2+} uptake upon Ca^{2+} restoration [52]. In order to take advantage of the protective effect of reduced temperature, the initial washing steps, which are usually performed in Ca^{2+}-free solution, may be performed at room temperature [15,53]. However, the narrow optimum temperature of digestive enzymes around 37 °C limits the value of this approach for the cell isolation procedure. Isolation of rat ventricular myocytes at 20 °C has been reported [54] and a method has been described for isolating human atrial myocytes at 30 °C [55]. Nevertheless, the vast majority of laboratories perform enzymatic digestion at 35–37 °C (Supplementary Tables 2 and 4; large mammals and humans respectively).

It has been suggested that a reduction of the pH to 6.8 increases the time period of Ca^{2+}-free perfusion above which reintroduction of Ca^{2+} evokes the Ca^{2+} paradox [56]. It is assumed that the high extracellular H^+ concentration favours the extrusion of intracellular Na^+ by the Na^+/H^+-exchanger and thereby reduces the Na^+ load. In accordance, several groups use the beneficial effects of a lower pH during perfusion of tissue with low-Ca^{2+} solution [55,57].

4.3. Na^+ concentration and osmotic pressure

Since Na^+ overload may play a crucial role in the pathophysiology of the Ca^{2+} paradox, it has been suggested that decreasing Na^+ in the Ca^{2+}-free solution could also prevent the Ca^{2+} paradox [48]. Following this hypothesis Gintant [58] and Hatem et al. [59] used Ca^{2+}-free solutions without Na^+ or with a low Na^+ concentration, respectively.

As discussed above, one factor likely to contribute to the Ca^{2+} paradox is the increased sarcolemmal permeability to electrolytes and/or water, leading to excessive osmotic swelling upon the return of Ca^{2+}. Reducing the Na^+ concentration in the Ca^{2+}-free solution may further exacerbate the osmotic swelling. In contrast, it has been shown that increasing the extracellular osmotic pressure can attenuate cell damage during Ca^{2+} repletion [60]. Commonly used for this purpose are colloids such as BSA [61], sugar alcohols such as mannitol [36] and sugars such as sucrose, raffinose and saccharose [37]. Glucose is often used, but is not generally ideal since it can be taken up by cells and metabolised.

4.4. Taurine and creatine

Isolation of myocytes leads to the loss of specific cellular compounds, and there may be benefit in supplementing these in the perfusing solutions. Taurine is a highly prevalent amino-sulfonic acid with a diverse range of physiological effects on the heart, retina, skeletal muscle, and central nervous system [62]. Kramer et al. [63] showed that intracellular taurine concentrations decrease during development of the Ca^{2+} paradox. When 10 mmol/L taurine was applied to isolated rat hearts during the Ca^{2+}-free period, a dramatic increase in recovery of mechanical function and a decrease in loss of nucleotides and creatine phosphokinase was seen. It is assumed that high intracellular levels of taurine (either due to taurine uptake or reduced taurine loss in taurine-containing solutions) activate a sarcolemmal taurine/Na^+-symporter which limits the rise of intracellular Na^+ during the Ca^{2+}-free period [64]. Inclusion of taurine in the Ca^{2+}-free solution is therefore presumed to have beneficial effects on the Ca^{2+} tolerance of isolated cardiac myocytes [24–33,65,66].

Similarly, a loss of cellular creatine has been reported during isolation of mouse ventricular myocytes [67]. When creatine was added to digestion buffers, cellular viability and performance were enhanced. Supplemental creatine has also been used empirically during the isolation of canine atrial myocytes [58].

4.5. Uncoupling agents

One of the main determinants of the Ca^{2+} paradox is extensive Ca^{2+} influx into the cytosol, which is paralleled by a massive cellular contraction. Contraction-induced sarcolemmal disruption may be responsible for loss of intracellular components and thereby play a major role in the detrimental effects of the Ca^{2+} paradox [46]. 2,3-butanedione monoxime (BDM) uncouples excitation–contraction by inhibiting ATPase activity of myosin II [68]. Adding BDM during the last 2 min of Ca^{2+}-free perfusion reduces the loss of intracellular components upon Ca^{2+} reintroduction, suggesting a cardioprotective effect of this drug [69]. However, BDM has various side effects, such as nonspecific phosphatase activity, which leads to alterations in the action potential, altered Ca^{2+}-handling and depletion of cellular ATP stores [70,71]. This may hamper its general usage during isolation of atrial myocytes.

Blebbistatin, a myosin II inhibitor, is widely used for excitation–contraction uncoupling in optical mapping. No significant effects of blebbistatin on the electrocardiogram, electrical conduction, or action potential waveform have been reported in the mouse [72] and rat [73]. Other groups, however, have reported blebbistatin-induced action potential prolongation in Langendorff-perfused rabbit ventricle [74]. Blebbistatin has been used as an alternative to BDM [75].

5. Enzymatic digestion

Isolation of single cells requires breakdown of the extracellular matrix (ECM) which is the structural scaffold supporting myocytes. Apart from the mechanical (trituration) and chemical (Ca^{2+} omission, use of chelating agents) methods of ECM breakdown, the use of tissue-dissociating enzymes represents the most important determinant of cell dissociation.

The ECM comprises a fibrous component, the main constituents of which are types I and III collagen [76] but also includes elastin. This is complemented by an amorphous "ground substance" containing hydrophilic polysaccharides (e.g. glycosaminoglycans) and multi-adhesive glycoproteins such as laminin or fibronectin, which provide strength to the tissue by binding to integrins on the surface of myocytes [77].

The ability of collagenases to digest heart tissue is well known [78]. It is notable that diseases such as heart failure or atrial fibrillation result in remodelling of the ECM where collagen abundance, type, and structure can be altered (for review see [2,79,80]) and thus cell isolation techniques require adaptation to given disease states.

Many investigators use a combination of collagenases and a general protease to isolate cells from mammalian atria. The idea that both were essential to produce viable cells was raised by Kono et al. [81], who in 1969 successfully isolated rat ventricular myocytes using a combination of collagenase, trypsin and chymotrypsin. Isolation was unsuccessful when either a mixture of purified collagenases alone or when a mixture of trypsin and chymotrypsin were used. These findings suggested that cardiac extracellular matrix contains two components: the first is digestible with purified collagenases, and the second digestible with a general protease. It has therefore been thought that collagen's triple helical structure is resistant to proteolytic damage. The unique ability of collagenases to crack the collagen structure has been thought necessary to initiate digestion [82], and only after this initial cleavage are the collagen fragments accessible to hydrolytic digestion by other less specific proteases. Other ECM macromolecules (i.e., glycoproteins, proteoglycans) are, at this time, degraded by neutral protease activity [83]. However, protocols using a primary digestion with protease, only later employing collagenase have been described [84], as have protocols using collagenase alone [85,86].

5.1. Collagenase preparations from Clostridium histolyticum

Commercially available "collagenases" are usually purified from supernatants of Clostridium histolyticum cultures. These "collagenases" are crude preparation containing more than 30 different enzymes including casein-digesting proteases, cellular debris, pigments and endotoxins [87]. Seven forms of collagenase have been purified from Clostridium, which fall into two classes [88]. Studies on substrate specificity revealed that class I collagenases encoded by the colG gene prefer natural substrates such as intact collagen whereas class II collagenases encoded by colH seem to act preferentially on short synthetic substrates [89]. Highly purified collagenases have been shown to be of limited use for the isolation of cardiac myocytes when applied alone [81], instead requiring additional proteases to achieve respectable cell yields.

In contrast to vertebrate collagenases, clostridial collagenases can make multiple cleavages within the triple helical regions and can also degrade denatured collagen [88]. Conversely, vertebrate collagenases initiate hydrolysis of collagen by making a single cleavage through all three chains after which other proteases then carry out the hydrolysis of the denatured collagen fragments (gelatinase activity). In addition, clostridial collagenases can digest almost all types of collagen, making them the enzymes of choice for digestion of extracellular tissue and cell dissociation.

Collagenase preparations exhibit a high batch-dependent variability in their precise enzyme composition and suppliers group each batch into distinct collagenase "types". These classifications are based on measurements of collagenase activity and behaviour of other proteolytic

enzymes such as caseinase, clostripain and trypsin. Since there is no general agreement concerning specifications of collagenase "types", these types are hardly comparable between different suppliers. Batch-to-batch variability in enzyme activities (see above) requires newly acquired batches to be tested at different concentrations. This is perhaps the most critical determinant of successful cardiomyocyte isolation. Samples can be obtained from manufacturers at no cost.

An in-depth description of the various assays to determine these enzymes' activities is beyond the scope of this review and we refer the interested reader to recent publications covering this issue [83].

A relatively new approach of suppliers to resolve the batch-dependent variability is the introduction of enzyme preparations consisting of blends of highly purified collagenase activities. This type of enzyme has been successfully used to isolate atrial cells from mouse [90], rabbit [91] and rat [92]. However, Kaestner et al. [23], isolating human atrial myocytes, directly compared a purified collagenase blend (Blendzyme 4, Roche) with the more commonly used crude collagenase mix. They reported that Blendzyme produced poor cell yields, an outcome that we have also found in our own experience.

5.2. Proteases

Proteases have been found to aid myocyte isolation in the hands of most [81] if not all investigators [86,93]. Trypsin is a serine protease which cleaves proteins and peptides within the amino acid chain (endoprotease) on the C-terminal side of lysine and arginine amino acid residues. Its pH optimum lies between 7.5 and 8.5 and its temperature optimum is at 37 °C [94], making this enzyme suitable for cell isolation. Trypsin was used in the earliest reports of cardiomyocyte isolation [95], and is used by several groups for isolation of human atrial myocytes [96], although to the best of our knowledge has not been used to isolate atrial cells from other large mammalian species.

Other proteases are commonly used for isolation of both human and large mammalian atrial myocytes, such as subtilisin Carlsberg (Protease XXIV, former Protease VII, Sigma-Aldrich) obtained from *Bacillus licheniformis* [97]. Similar to trypsin, subtilisin Carlsberg is a serine endoprotease with pH-optimum of 7.5. It retains its activity at higher temperatures and is widely used in modern laundry detergents. Other groups use "pronase E" (Protease XIV, Sigma-Aldrich) derived from *Streptomyces griseus* [39]. Pronase E is non-specific containing at least 10 proteolytic enzymes [98]. The optimum pH for pronase E is between seven and eight and it has a wide range of working temperature. While inclusion of such proteases aids cell isolation, other proteins such as ion channels may also be degraded, specifically those carrying the K^+ current I_{Kr} [99].

Cardiac ECM also contains elastin fibres and elastase is the only neutral protease which is able to digest this protein. Some investigators have therefore found it useful to include elastase in the isolation of human atrial myocytes [49]. Elastase has not been used to obtain atrial myocytes from other large mammalian species, with the exception of cells from the sinoatrial node [100].

6. Myocyte storage solutions

After isolation, freshly dissociated and washed atrial myocytes are usually transferred to a "storage solution" in which they remain until usage. For this purpose modified versions of the Ca^{2+}-free solution or physiological salines may be used. We and others stored myocytes in solutions containing EGTA in high concentrations (10 mmol/L) to avoid reintroduction of Ca^{2+} into myocytes and prevent the Ca^{2+} paradox [24–29,101]. The EGTA was washed out before experimentation began. However, the authors found that even brief EGTA exposure impairs Ca^{2+} transient measurements even after a long (>30 min) washing period with EGTA-free Ca^{2+}-containing (2 mmol/L) bath solution [31–33].

As an alternative to using EGTA, storage solutions may instead contain small amounts of Ca^{2+} at concentrations somewhat lower than the physiological range. This provides a gradual reintroduction of Ca^{2+} before experimentation begins. BDM may be added to uncouple excitation–contraction coupling and thereby protect the myocytes from the Ca^{2+} paradox.

Isenberg et al. incubated rat and guinea pig ventricular myocytes for at least one hour in medium containing high K^+ but reduced Na^+ concentrations in order to normalise intracellular Na^+/K^+ ratios [102,103] before reintroducing Ca^{2+}. Isenberg et al. designated this solution "KB-medium" from the abbreviation of the German word Kraftbrühe for "power soup". In addition, KB-medium usually contains supplemental ATP to help maintain a normal Na^+/K^+ ratio via the sarcolemmal Na^+/K^+ pump, as well as maintaining physiological Ca^{2+} levels via sarcolemmal and sarcoplasmic reticulum Ca^{2+} pumps. Substrates for the glycolytic (glucose) and oxidative (pyruvate) pathways, as well as for oxidative phosphorylation (succinate, β-OH-butyrate) are added, to provide energy for Ca^{2+}-activated mitochondrial metabolism. KB-solution is used to store human [37,104] and large mammalian [105,106] atrial myocytes.

7. Evaluation of myocytes quantity and quality

When establishing an isolation procedure and also when testing a new enzyme batch (see above) it is important to evaluate the quantity and quality of the myocytes that have been isolated. Cell yield can be quantified crudely by counting the healthy myocytes in a 10 μl sample. A microscopic slide with an engraved grid or a Neubauer chamber may be of help.

Although yield is a good indicator of a successful isolation, other measures of cell quality can be used. The simplest marker of a high-quality myocyte is a square-edged rod-shaped appearance with clear striations. Myocyte health can be further assessed by the cells' ability to exclude a dye such as trypan blue. It is important to note that this method is not completely reliable since myocytes, which have taken up the dye, can still recover from the stresses of isolation and survive well in culture [9,107]. Finally it is important to evaluate the isolation procedure based on the experimental parameters, which are studied in the myocytes and based on the specific needs of the experimental question.

It may be assumed that different isolation methods, specifically chunk versus perfusion method (see below), can lead to a greater or lesser loss of cellular constituents such as t-tubules or a specific ion channel such as I_{Kr} [16]. Using Ca^{2+} buffers such as EGTA during the isolation procedure can impair cellular Ca^{2+} transient measurements and recordings of cell shortening. Accordingly, in our hands, including EGTA in the storage solution for human atrial myocytes produced artificial biphasic Ca^{2+} transients in response to a depolarizing voltage step (Fig. 1) [108]. The biphasic form could still be observed even after a 10 minute perfusion with EGTA free bath solution. Similarly, BDM may be omitted if single cell contractility is of central importance. Response to field stimulation may be an additional indicator of viable atrial myocytes. However, this parameter might be of limited value in remodelled cells, because impaired response to electrical activation may be a component of the remodelling process.

8. Step-by-step procedures

8.1. Isolation of single atrial myocytes from large animals

8.1.1. Use of the perfusion method

Similarly to other laboratories we have found that when tissue is not limited to small samples or biopsies, as is often the case with human tissue, a retrograde perfusion system (with solutions flowing retrogradely down the aorta, as opposed to normal anterograde physiological flow) employing the use of a Langendorff apparatus is preferable in terms of

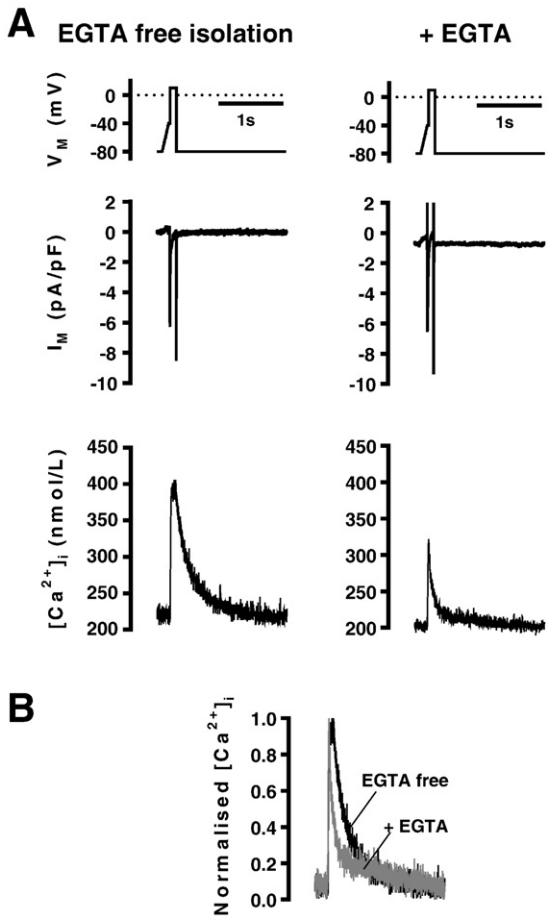

Fig. 1. Effect of EGTA during cell isolation on systolic Ca^{2+} transient in human atrial cardiomyocytes. A. Representative recordings of L-type Ca^{2+} current and cytosolic Ca^{2+} transients in response to a depolarising voltage pulse (whole-cell voltage-clamp technique). EGTA was excluded (left panel) during cell isolation or included in the storage solution (right panel). In both cases, experiments were started after a 10 minute perfusion with EGTA-free bath solution. B. Normalized traces for both conditions. For detailed methods see [31,32].

cell quality and reproducibility. Differences have been seen between myocytes isolated using different techniques: when an arterially perfused method of atrial myocyte isolation was directly compared with chunk digestion in dogs, delayed rectifier K^+ currents were recordable from perfusion isolated myocytes but not from those isolated by chunk digest [16]. While the chunk digest method is used by some [57,84], the perfusion method is more commonplace. The specific protocol varies between laboratories (Supplemental Table 2). We will discuss methods below, comparing and contrasting those used in different laboratories and species. Details of the specific protocol we use for isolation of atrial myocytes from the sheep can be found in the Supplemental Materials.

8.1.2. Equipment and preparation

The basic equipment required is shown in Fig. 2A. Two physiological solutions are used. The composition of the solutions used by our group can be found in Supplemental Table 1, and those of other groups in Supplemental Table 3. A Ca^{2+}-free [109], low-Ca^{2+} [61] or cardioplegic solution [35] is used for initial washing and enzyme perfusion. A taurine-containing low-Ca^{2+} solution is used for final washing and tissue dissociation. The solutions are stored in reservoirs warmed directly by a water bath. The warmed solutions pass, via silicone rubber tubing, to a 3-way tap for easy solution exchange. While our laboratory uses a peristaltic pump to drive solutions at constant flow rate to a Langendorff coil, other groups use a constant pressure gravity-fed system. Some groups equilibrate their solutions with oxygen or 5% carbogen [110] (Supplemental Table 2), but we have not found this necessary in the isolation of ovine atrial myocytes.

The core of the setup is a Langendorff coil. The outer jacket is warmed by the water bath, set at 37 °C. Physiological solutions flow down through the coil until reaching continuity with the base of a central column that functions as a bubble trap. The central column exits downwards to a cannula.

A cannula is attached to the Langendorff coil via another 3 way tap using silicone rubber tubing. Cannulae are commercially available or may be fashioned in-house. We use stainless steel rodent gavage feeders or alternatively nylon peripherally inserted central venous cannulae that have been cut down. A key feature is the presence of a terminal lip that facilitates a tight seal with the target vessel by allowing the cannula to be retracted to the level of the ligature (Fig. 2B).

In order to preserve enzyme containing solution, a recirculation line driven by the peristaltic pump collects perfusate from the tissue and returns it to the solution reservoir.

In preparation for cell isolation, the Langendorff coil, cannulae and all tubing are autoclaved. The equipment is assembled and all lines are flushed with distilled water. The tubing is then primed with Ca^{2+}-free and taurine-containing solutions, taking care to remove air bubbles. The central column of the Langendorff is filled with Ca^{2+}-free solution and stoppered. Sutures, syringes and scissors are made readily available.

8.1.3. Heart extraction and cannulation

An example isolation protocol is shown in the flowchart (Fig. 3) with links to the figure numbered in the text. Two minutes prior to sacrifice, a bolus of 20,000 IU of heparin is administered via a peripheral venous cannula to reduce the chances of thrombus formation which could jeopardise perfusion. Following euthanasia with sodium pentobarbitone (typically 1 mL/kg of a 20% solution administered intravenously), the heart is removed via thoracotomy and dissected free at the level of the ascending aorta (1). At this point, some groups inject cardioplegic solutions into the coronary arteries [34–36]. The heart is then washed in Ca^{2+}-free solution. Minimising the time between euthanasia and cannulation preserves cellular viability.

Approaches to cannulation differ between laboratories. In smaller subjects, direct cannulation of the aorta is used (See Louch et al. for review [9]). In larger species, some groups remove the tissue of interest and cannulate a vessel within the specimen itself. In our group we have had most success from cannulating the coronary arteries directly.

The atria are dissected from the ventricles 2 cm below the atrioventricular groove. The aortic root is identified and the Sinuses of Valsalva visualised. If left atrial myocytes are required, the ostium of the left main coronary artery is dissected. Although coronary anatomy varies considerably, in 60–70% of cases a coronary branch to the left atrial appendage can be identified 1–3 cm distal to the ostium of the left mainstem. A cannula is passed into the vessel and secured using 2–0 silk (2). The cannula is retracted until it rests snugly against the ligature. If such a branch is not found, the circumflex coronary artery which runs along the atrioventricular groove is cannulated and secured in the same manner. Similarly, if right atrial myocytes are required, the cannula is placed within the ostium of the right coronary artery.

Once the cannula has been sited, perfusion is checked using a 50 mL syringe filled with Ca^{2+}-free solution. If the circumflex or right coronary arteries have been used, cut vessels are ligated using crocodile clips or sutures. If perfusion is good, the atrial appendage should blanch. If perfusion is poor, the cannula should be manipulated to ensure it is fully retracted against the ligature, and that the optimal angle between cannula and vessel has been achieved. Ligated vessels should also be inspected for leakage.

The 50 mL syringe is disconnected, and the tissue connected to the Langendorff coil and supported on a collecting vessel. The flow rate is adjusted to the minimum that achieves good perfusion, marked by tissue blanching, warmth and slight swelling. Any barriers to good

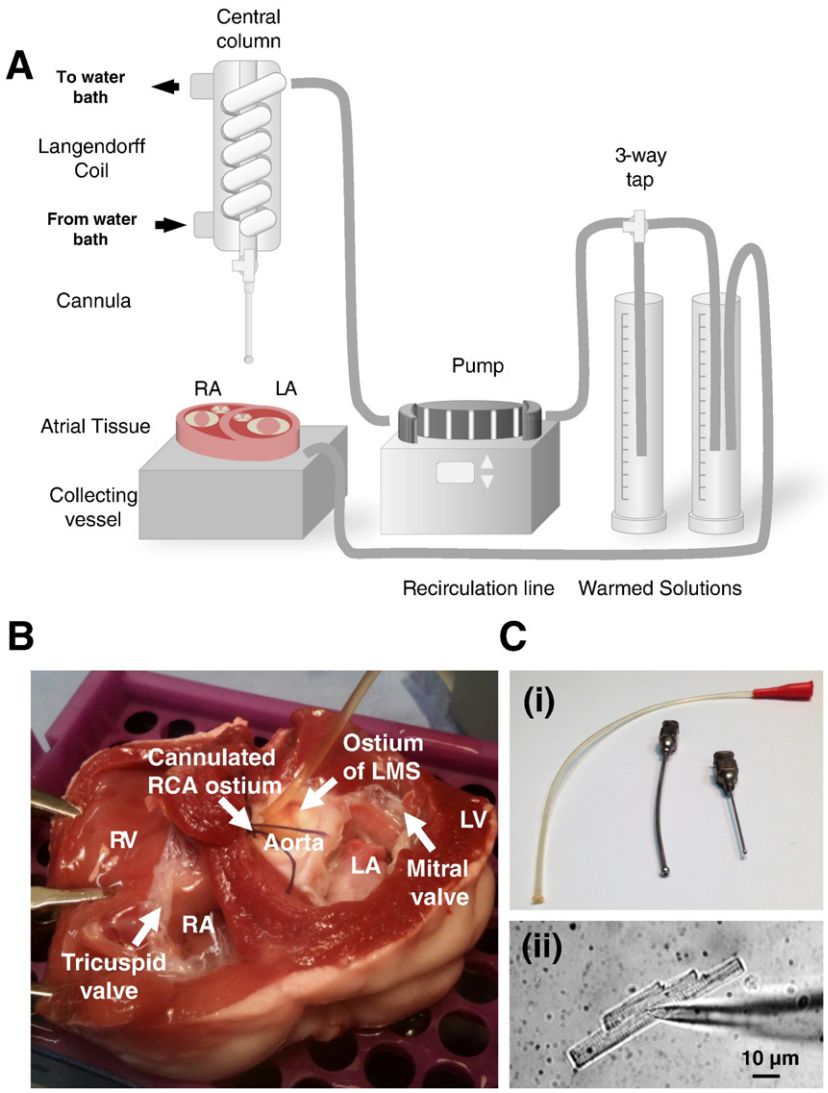

Fig. 2. A. Isolation setup for the perfusion method of myocytes from atria of large mammals. B. Atrial tissue specimen with nylon cannula perfusing right atrium via right coronary artery. Note clips on cut edge of ventricle to prevent excessive leakage of solutions. C. (i) Examples of cannulae used for atrial isolation in large mammals. (ii) Left atrial myocyte from sheep with patch pipette. LA — left atrium, LMS — left main stem, LV — left ventricle, RA — right atrium, RCA — right coronary artery, RV — right ventricle.

perfusion such as leaks are rectified. In our laboratory we use a 10 minute wash period without recirculating solutions before applying enzymes (3). During this time the surface of the tissue is gently rinsed with Ca^{2+}-free solution to remove any remaining blood.

8.1.4. Enzyme application

Following the wash period, enzyme is added to the Ca^{2+}-free solution (4). Depending on flow rate, 2–3 min elapses allowing the enzyme to reach the tissue. After this, the perfusate is collected and recirculated back to the reservoir to preserve enzyme. Several stages of tissue digestion are observed. The perfusate on the surface of the atrium which is initially watery becomes viscous and the tissue becomes turgid. As digestion progresses, translucent regions appear in the appendage and the tissue becomes flaccid again. The optimum digestion time is influenced by the type and concentration of enzyme use, and also varies between disease models and individual subjects. In our group we adjust the enzyme concentration so that a 12-minute digest on average yields viable atrial myocytes from a control sheep. The optimal digestion time is generally 20% longer in our model of tachypacing-induced heart failure compared to control due to the atrial collagen deposition seen in this model. Once digestion has progressed satisfactorily, the perfusing solution is exchanged for a taurine-containing [109] or KB [106] solution. We employ a 20 minute wash period to remove residual enzyme (5).

After washing, the atrial appendage is dissected from the remaining tissue and chopped into 1 mm^3 chunks (6). The tissue chunks are resuspended in ~30 mL of taurine solution and agitated using a Pasteur pipette (7). After three minutes of agitation, 15 mL of the supernatant is collected via filtration through a nylon mesh into a test tube. 15 mL of fresh taurine solution is added to the tissue suspension. This is repeated until 8–10 tubes have been collected. After a settling period, cell pellets form (8). The supernatant is discarded and the cell pellets reconstituted with 3 mL of a solution containing a higher concentration of Ca^{2+}. We use a 50:50 mixture of taurine solution and modified Tyrode's solution for this purpose, although other groups employ KB medium [53,106].

8.2. Isolation of single atrial myocytes from humans

8.2.1. Obtaining and transporting tissue

Although in principle whole human hearts can be perfused using a Langendorff perfusion system [111] we are not aware of any study that has used the perfusion method to obtain human atrial myocytes. To the best of our knowledge, all published studies use the so called chunk-method to obtain human atrial myocytes, which is described below.

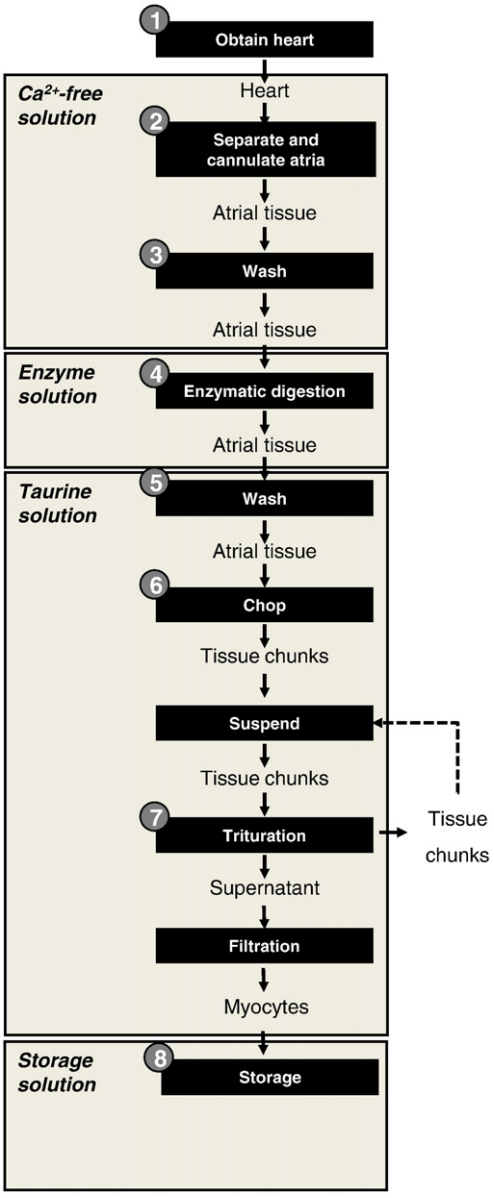

Fig. 3. Flowchart for example of perfusion method of isolation of atrial myocytes from large mammals.

During routine open-heart surgery with extracorporeal circulation, the tip of the right atrial appendage is usually removed and may be used for isolation of atrial cardiomyocytes. In patients undergoing mitral valve replacement, the tip of the left atrial appendage may also be obtained and used for the isolation of left atrial myocytes [29,55]. The surgical technique used to obtain the atrial sample influences the cell yield. We found that clamping the right atrial appendage prior to its removal was associated with an 80% reduction in cell yield when compared with resecting the appendage with a purse-string suture only (Fig. 4A). The mass of atrial appendage removed is also important. We found that tissue samples weighing between 150 mg and 450 mg produced the highest cell yields, while samples lying outside this range generated fewer cells (Fig. 4B).

Results obtained from human samples usually have higher variability compared to standardised animal models. This may be at least partially due to patients' characteristics: underlying heart disease, systemic disease or medication (Supplemental Table 6). We analysed cell-yields obtained using our standard isolation protocol which included EGTA in the myocyte storage solution [25–28,30]. Analysis of covariance (ANCOVA) with stepwise variable selection revealed that chronic AF was associated with decreased cell yield while treatment with dihydropyridine Ca^{2+}-channel blockers was associated with increased cell yield. These two factors accounted for ~23% of variability in cell yield (Supplemental Table 7). Inclusion of other patient characteristics (Supplemental Table 6) did not improve the model significantly. This suggests that other features which were not represented in the patients' recorded characteristics, such as lifestyle and genetics, may also contribute to cell yield.

We assume that in patients treated with Ca^{2+}-channel blockers, reduced cytosolic Ca^{2+}-uptake after reintroduction of Ca^{2+} during the isolation procedure may attenuate the detrimental effects of the Ca^{2+} paradox (see above) thereby increasing cell yield [112]. Similarly, it is conceivable that impaired cytosolic Ca^{2+} in patients with cAF may contribute to reduced cell yield [31,32,37,65]. However, other mediators such as increased fibrosis and apoptosis may be possible [113].

8.2.2. General procedure for isolation of human atrial myocytes

Although most research laboratories have developed their own isolation methods, there is a common thread which runs through most published protocols. Within this section we will describe the general procedure for human atrial myocyte isolation and will mention possible modifications. As before, a linked flowchart of the procedure is shown in Fig. 5. Differences between groups in the solution compositions and enzymes used are shown in Supplemental Tables 4 and 5. A visualised version of the protocol described here is available online [114].

After excision (1) the tissue sample is transferred immediately into a sterile transport medium. This is usually based on a cardioplegic solution in order to reduce energy and oxygen consumption, and prevent accumulation of toxic metabolites (see above).

During the first step of the isolation procedure fat and connective tissue are removed from the tissue and the sample is chopped into small chunks of approximately 1 mm^3 in size (2). Using a blade instead of scissors may be advantageous, probably because of the mechanical damage caused by tugging of the tissue with scissors [115].

Washing the tissue chunks is optional during the isolation process (3). Although in our experience omitting the washing step does not influence cell yield, washing the tissue chunks largely removes remaining blood and results in a clear reduction in erythrocyte count and debris in the final preparation (Fig. 4C). This may be of particular importance in patch-clamp experiments, where soilings may potentially stick to the pipette tip and impair seal formation. Washing is usually performed by transferring the tissue chunks into a washing buffer and stirring carefully for about 5 min. This step may be repeated 1–2 times.

There is variation between published protocols concerning the buffers used for these first steps. Whereas most groups chop the tissue in transport solution and use Ca^{2+}-free solution as a washing buffer, Hatem et al. [59] performed chopping and washing in BDM-containing transport medium. It is assumed that BDM prevents cutting injury pointing to a possible advantage of chopping the tissue in BDM-containing transport medium [116]. However, we and other groups use Ca^{2+}-free solution for both chopping and washing the tissue [24–33, 117], indicating that there is no clear disadvantage to directly transferring the tissue to Ca^{2+}-free solution before starting with the preparation.

For enzymatic digestion, the chopped tissue is placed in a jacketed reaction beaker containing a magnetic stirring bar (Fig. 4D). The beaker is warmed to 37 °C and gassed with 100% O_2. During the first digestive step, the tissue is stirred for 30–45 min in enzyme solution (4), based on Ca^{2+}-free solution and containing digestive enzymes such as collagenases and proteases (see above). Although the supernatant may already contain viable cells, a second enzymatic digestion is usually performed to further increase cell yield.

The tissue is transferred into fresh enzyme solution (5), usually containing collagenases alone. The second digestion is either performed for a fixed amount of time, established experimentally, or the cell yield of the supernatant is tested every 2–3 min during the second digestion step until rod-shaped, striated cardiomyocytes appear. To further

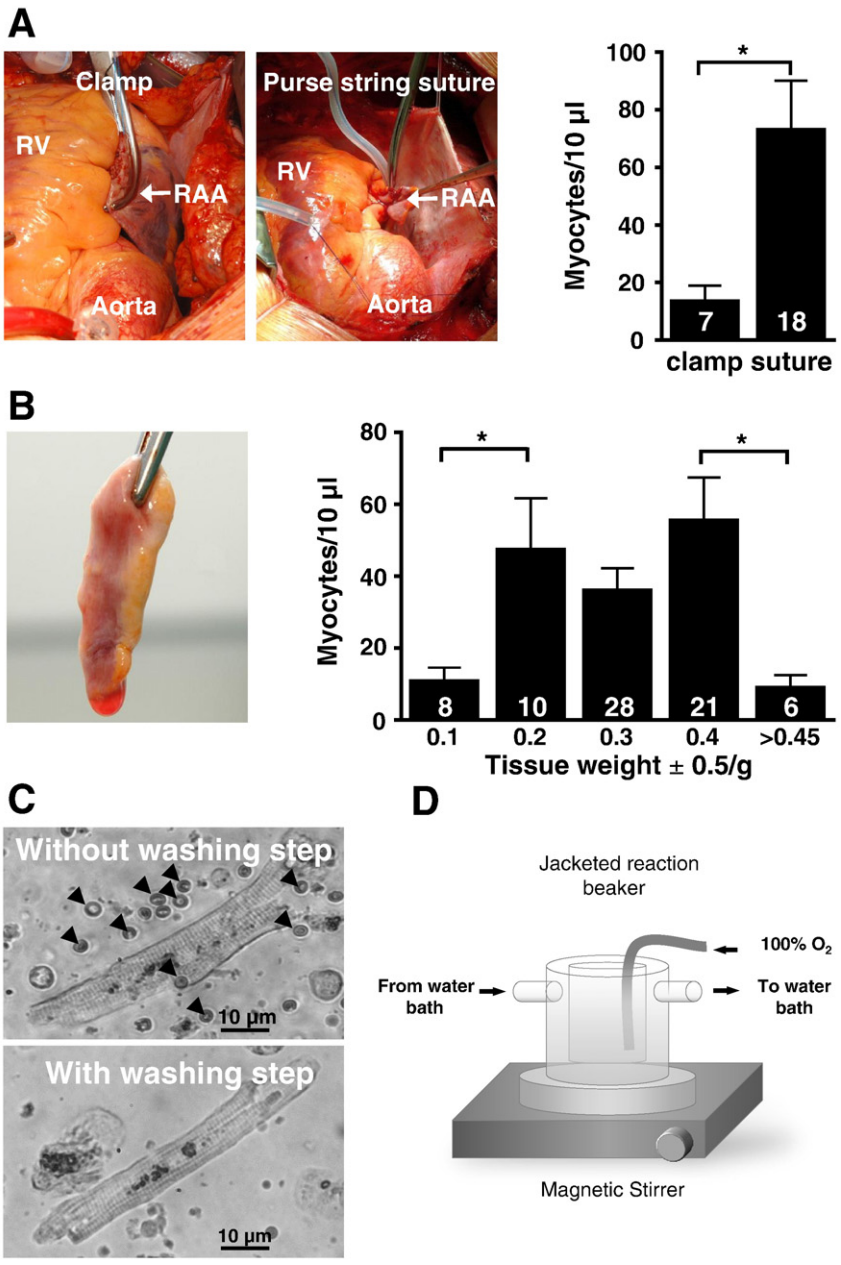

Fig. 4. A. Surgical view of atrial tissue dissected using clamp or purse string suture. Increased cell yield is seen if purse string suture is used. B. Right atrial appendage specimen. Optimal tissue weight is 0.2–0.4 g. C. Examples of atrial myocytes with and without washing steps. Arrowheads indicate erythrocytes. D. Equipment for isolation of myocytes from human atrial tissue. RAA — right atrial appendage, RV — right ventricle.

increase cell yield the tissue chunks may be triturated gently after each digestion step using a Pasteur pipette (6). The second digestion step may be repeated several times with the remaining tissue chunks, while the supernatants are kept until final centrifugation.

To remove large undigested chunks of tissue, the cell containing supernatant is filtered through a 200 µm nylon mesh (7). Finally, cells are separated from the supernatants by gentle centrifugation (95 g for about 10 min) and the precipitate is resuspended in 4 mL of the storage solutions described above (8).

9. Conclusions

Cellular dissociation requires a combination of mechanical, chemical and enzymatic approaches to yield viable cells. The protocols detailed above may be adapted to the specific needs of the experimental question. BDM may be omitted if single cell contractility is of central importance. Alternatively, the use of chelating agents such as EGTA may be minimised if Ca^{2+} cycling is the primary focus [31–33]. Washing of cells may not be required if Ca^{2+} transients are studied alone but may be of benefit if the cells are to be patch-clamped.

Isolation processes have been iteratively improved over the last three decades and experimental material from human and large mammalian subjects is now frequently obtained. The use of isolated cardiomyocytes provides the unique opportunity to study the cellular electrophysiological properties that predispose to, precipitate and perpetuate AF while eliminating interactions between cells. The next decades will see these techniques used to further our understanding of atrial physiology in health and disease.

Disclosures

None.

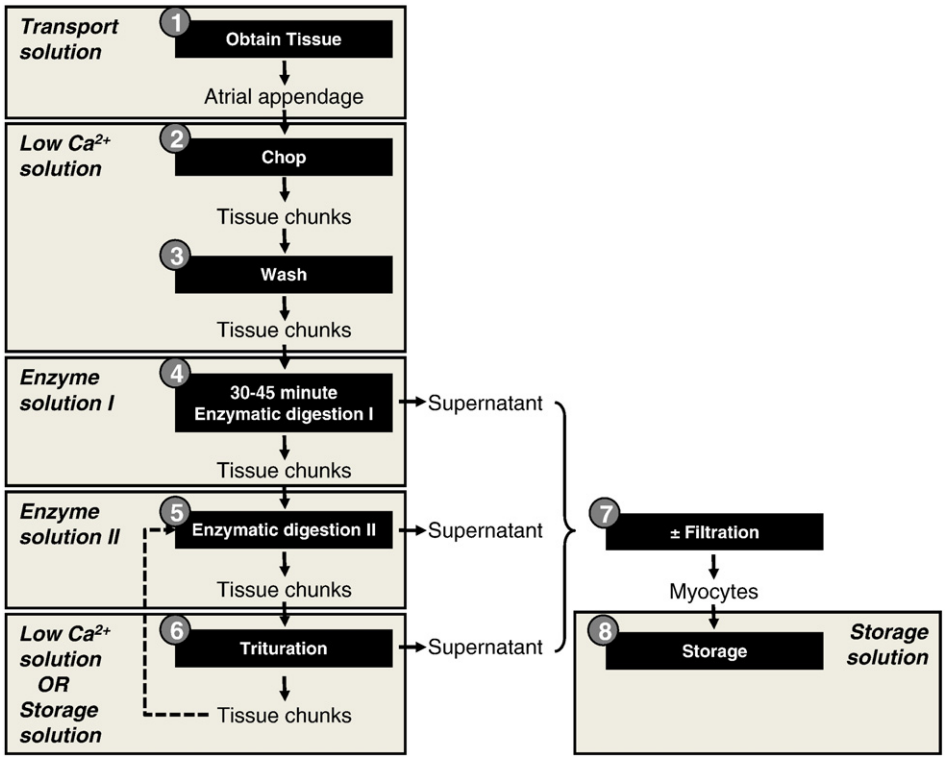

Fig. 5. Flowchart of chunk digest method for isolation of atrial myocytes from human atrial tissue.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.yjmcc.2015.07.006.

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